

## Mutations affecting predation ability of the soil bacterium *Myxococcus xanthus*

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*Myxococcus xanthus* genetic mutants with characterized phenotypes were analysed for the ability to prey on susceptible bacteria. Quantification of predatory ability was scored by a newly developed method under conditions in which prey bacteria provided the only source of nutrients. These results were corroborated by data derived using a previously published protocol that measures predation in the presence of limited external nutrients. First, early developmental regulatory mutants were examined, because their likely functions in assessing the local nutrient status were predicted to be also important for predation. The results showed that predation efficiency is reduced by 64–80 % for mutants of three A-signalling components, AsgA, AsgC and AsgE, but not for AsgB. This suggests that an Asg regulon function that is separate from A-signal production is needed for predation. Besides the Asg components, mutations in the early developmental genes *sdeK* and *csgA* were also consistently observed to reduce predatory efficacy by 36 and 33 %, respectively. In contrast, later developmental components, such as DevRS, 4406 and PhoP4, did not appear to play significant roles in predation. The predatory abilities of mutants defective for motility were also tested. The data showed that adventurous, but not social, motility is required for predation in the assay. Also, mutants for components in the chemotaxis-like Frz system were found to be reduced in predation efficiency by between 62 and 85 %. In sum, it was demonstrated here that defects in development and development-related processes affect the ability of *M. xanthus* to prey on other bacteria.

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## INTRODUCTION

*Myxococcus xanthus* is a ubiquitous, soil-residing predatory bacterium, which moves in colonial swarms during the vegetative part of its life cycle (for a review, consult Dworkin, 1996). It is thought that the collective secretion of biocidal factors by the swarm enhances the colony's predation of a wide range of susceptible microbial prey, including firmicutes, proteobacteria like *Serratia marcescens*, and both bacterial and fungal phytopathogens (for a review, consult Rosenberg & Varon, 1984). Although *M. xanthus* growth can also be sustained by adequate concentrations of certain amino acids and central metabolic intermediates, predation appears to be an integral part of the *M. xanthus* survival strategy when such nutrients become limiting. The major characteristics of *M. xanthus* predation – mobility and collective group action – set these organisms apart from other bacterial predators with tractable genetic systems, and hence establish them as a suitable model for studying this particular mode of predatory behaviour.

Predation, however, represents only part of the *M. xanthus* life cycle. In the absence of prey or suitable nutrients, the

*M. xanthus* cells within a dense, starving colony cooperate to form a macroscopic fruiting body, within which 1–10 % of the cells differentiate into resistant myxospores (Dworkin, 1996). The execution of this developmental program is dependent on a number of regulatory components implicated in the sensing of environmental signals, including the developmental regulators AsgA (Plamann *et al.*, 1995), CsgA (Kim & Kaiser, 1990; Shimkets & Rafiee, 1990) and SdeK (Garza *et al.*, 1998; Kroos *et al.*, 1990).

In addition, since the formation of fruiting bodies is dependent on the movement of cells towards aggregation centres, mutations in many motility genes also cause deleterious effects on development (Hodgkin & Kaiser, 1979a, b; MacNeil *et al.*, 1994b; Zusman, 1982). *M. xanthus* possesses two separate motility mechanisms, termed adventurous (A) motility and social (S) motility. The A-motility system mediates the spatial translocation of both individual and small groups of cells, while the S-motility system governs the contact-dependent movement and coordination of swarms of cells (Hodgkin & Kaiser, 1979a, b; MacNeil *et al.*, 1994a, b). S<sup>−</sup> mutations cause greater defects in fruiting-body morphology than mutations

in the A system, which suggests that proficiency for S motility is more important for development (MacNeil *et al.*, 1994a, b). In addition, the Frz system, which comprises components that are homologous to the Che proteins involved in chemotaxis of *Escherichia coli* (Blackhart & Zusman, 1986; McBride *et al.*, 1989; Trudeau *et al.*, 1996), regulates the developmental process by controlling the reversal frequencies of individual cells (Shi *et al.*, 1996).

Since local nutrient levels dictate entry into either the developmental or predatory modes of the *M. xanthus* life cycle, it is likely that mutations affecting entry into one mode might also impact entry into the other. Many techniques exist for determining the effect of various mutations on the organism's ability to go through the developmental cycle. As a result, a substantial body of information exists for the genetics of *M. xanthus* fruiting body formation, sporulation and motility (for reviews, consult Kaiser, 2003; Søgaard-Andersen *et al.*, 2003). Here, we contribute another piece to the proverbial puzzle by assessing the predatory capacity of mutants defective for various processes in both development and related motility systems.

In order to carry out this study, we developed a method for quantifying predation by *M. xanthus*. *S. marcescens* was selected as the primary prey species in a new assay described here, because it is (i) simple to culture, (ii) susceptible to lysis by *M. xanthus* (Singh & Yadava, 1976), (iii) easily imaged due to the striking contrast between its red

(prodigiosin-derived) pigmentation and the yellow colour of *M. xanthus* (Williams & Qadri, 1980), and (iv) accessible to other myxobacteriologists given its relatively low hazard potential to human health and agriculture (for a review, consult Hejazi & Falkner, 1997). In addition to this assay, in which predation by *M. xanthus* on live prey is obligatory for sustaining growth, we also employed a previously described assay that measures predation of prey bacteria in the presence of limited nutrients (Bull *et al.*, 2002). Both assays were used in this study since they measure predation under differing conditions, and, therefore, a comparison of their results might offer further insights into the genetic requirements for predation when such conditions are encountered in the soil.

METHODS

**Culture media and conditions.** A complete list of *M. xanthus* strains used in this study can be found in Table 1. *M. xanthus* strains were grown at 33 °C on CTTYE media (CTT containing 0.2% yeast extract) (Hodgkin & Kaiser, 1977). Kanamycin monosulfate (Sigma-Aldrich) was added to a final concentration of 40 µg ml<sup>-1</sup>, as needed. Table 2 lists the prey strains used in this study. *E. coli* and *S. marcescens* cells were cultured at 37 °C on LB media, unless otherwise indicated. Culture methods for phytopathogens were as previously described (Bull *et al.*, 1994; Cintas *et al.*, 2002; Goldman *et al.*, 2003; van Bruggen *et al.*, 1988).

**Lawn predation assay.** An overnight culture of *S. marcescens* ATCC 39006 was washed twice and resuspended in 7 volumes of TPM medium with a final OD<sub>600</sub> reading of about 0.8. Circles of

Table 1. List of *M. xanthus* strains

Strain	Genotype and other relevant information	Reference
DK1622	Wild-type	Kaiser (1979)
ASX1	DK1622 Δ <i>cglB</i>	Rodriguez & Spormann (1999)
DK1217	DK1622 <i>aglB1</i>	Hodgkin & Kaiser (1979a)
DK1250	DK1622 <i>aglB1 tgl-1</i>	Hodgkin & Kaiser (1979a)
DK3468	DK1622 <i>dsp-1680</i>	Shimkets (1986)
DK4294	DK1622 Tn5 <i>lac</i> Ω4406 (Km <sup>R</sup> )	Kroos <i>et al.</i> (1986)
DK5057	DK1622 Tn5-4560 <i>asgA476</i> (Tc <sup>R</sup> )	Kuspa & Kaiser (1989)
DK5208	DK1622 <i>csaA::Tn5-132</i> ΩLS205 (Tc <sup>R</sup> )	Kroos & Kaiser (1987)
DK5511	DK1622 Tn5-132 <i>lac</i> Ω4414 ( <i>devRS</i> ) (Tc <sup>R</sup> )	Thöny-Meyer & Kaiser (1993)
DK10410	DK1622 Δ <i>pilA</i>	Wu <i>et al.</i> (1997)
MS1512	DK1622 Δ <i>sdeK1</i>	Pollack & Singer (2001)
VP963	DK1622 Δ <i>phoP4</i>	V. D. Pham and others, unpublished results
DK101	<i>pilQ1</i> (leaky)	Hodgkin & Kaiser (1977)
DK480	DK101 <i>asgB480</i>	Hagen <i>et al.</i> (1978)
DK767	DK101 <i>asgC767</i>	Hagen <i>et al.</i> (1978)
MS2021	DK101 <i>asgE::Ω5003</i>	Garza <i>et al.</i> (2000)
DZ2	Wild-type	Campos & Zusman (1975)
DZ4478	DZ2 Δ <i>frzA</i> (codons 15–145)	Bustamante <i>et al.</i> (2004)
DZ4479	DZ2 Δ <i>frzB</i> (codons 11–92)	Bustamante <i>et al.</i> (2004)
DZ4481	DZ2 Δ <i>frzE</i> (codons 13–766)	Bustamante <i>et al.</i> (2004)
DZ4483	DZ2 Δ <i>frzF</i> (codons 13–582)	Bustamante <i>et al.</i> (2004)
DZ4484	DZ2 Δ <i>frzZ</i> (codons 16–280)	Bustamante <i>et al.</i> (2004)

**Table 2.** List of prey species

Species and strain	Habitat(s)	Reference
<i>E. coli</i> DH5 $\alpha$	Animal intestines; transiently in soil and water	Hanahan (1983)
<i>P. carotovorum</i> subsp. <i>carotovorum</i> JL1134	Soil; wide-host-range pathogen of plants	Bull <i>et al.</i> (1994)
<i>Pseud. syringae</i> pathovar <i>alisalensis</i> BS91	Phyllosphere; phytopathogen of crucifers; transiently in soil	Cintas <i>et al.</i> (2002)
<i>S. marcescens</i> ATCC 39006	Soil, water; opportunistic human pathogen	Bycroft <i>et al.</i> (1987)
<i>Sphing. suberifaciens</i> CA1	Soil; phytopathogen of lettuce	van Bruggen <i>et al.</i> (1988)
<i>X. campestris</i> pathovar <i>vitians</i> S339	Phyllosphere; phytopathogen of lettuce; transiently in soil	Goldman <i>et al.</i> (2003)

13 cm diameter nitrocellulose membrane (Bio-Rad) were briefly immersed in the cell suspension, placed atop CTTYE agar, and incubated at 33 °C overnight to allow the formation of even lawns of *S. marcescens*. To begin the predation assay, the nitrocellulose membrane with a confluent lawn of red-pigmented cells ( $1\text{--}2 \times 10^8$  cells  $\text{cm}^{-2}$ ) was transferred onto a starvation (TPM) agar plate, where the lawn was used as the sole source of nutrients for paired *M. xanthus* inocula. Mid-exponential-phase *M. xanthus* cells grown in CTTYE broth without selection were washed twice in TPM, and 20  $\mu\text{l}$  of cells from a genetic mutant strain and the wild-type parent strain were spotted at a concentration of  $5 \times 10^9$  c.f.u.  $\text{ml}^{-1}$  ( $1\text{--}2 \times 10^8$  cells  $\text{cm}^{-2}$ ) onto the same *S. marcescens* lawn approximately 6.5 cm apart. All comparisons between these paired *M. xanthus* strains, conducted on fourfold replicate lawns to assess experimental error, were made on the same nitrocellulose filter plate to remove filter-plate variability. After incubation at 33 °C for about 7 days, *M. xanthus* swarms were imaged with the FluorChem 8900 imager (Alpha Innotech) using white reflective light and the SYBR green filter – this filter was empirically determined to provide the best contrast – and the swarm size areas were quantified with the manufacturer's analytical software. The data are presented as an averaged swarm size of the test strain relative to that of wild-type *M. xanthus* strain DK1622. Each fourfold replicate experiment was conducted at least twice, and swarm size comparisons among these experiments were consistently reproducible.

Since differences in motility among the *M. xanthus* strains could affect predation results using the lawn assay, we devised the following method to account for potential motility differences. Swarming on nitrocellulose in the presence of non-prey nutrients – yeast extract and proteolytic digests of casein in this case – was determined by briefly immersing 13 cm circles of nitrocellulose membrane in CTTYE, then laying them atop CTTYE agar, and finally comparing these to assays conducted in the presence of prey bacteria (as described above). Wild-type and mutant strains were spotted onto the same nutrient filter, allowed to grow for about 7 days, and the swarm sizes imaged and quantified as above.

**Streak predation assay.** The streak predation assay was conducted according to previously published methods (Bull *et al.*, 2002). Briefly, bacterial cultures to be tested as prey (Table 2) were suspended in 100  $\mu\text{l}$  TPM buffer to a density of 0.6 OD<sub>600</sub> units, using a spectrophotometer (UV-1601, Shimadzu) to measure the latter. An inoculation loop was used to spread cells across a diluted casitone agar plate (dCTA) (0.2% casitone, 0.1%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.5% agar). Prey cells were spread in a 4 cm strip starting 1 cm from the edge of the medium. The liquid from prey culture inoculation was allowed to dry prior to inoculating the plates with myxobacteria. Myxobacteria were grown on dCTA and suspended in TPM to a cell density of 0.6 OD<sub>600</sub> units. A 20  $\mu\text{l}$  drop of the myxobacterial suspension was placed 0.5 cm from the beginning of the 4 cm strip of prey bacteria. The size of the bacterial strip and the

distance lysed was measured after incubation for 14 days at 33 °C. Each experiment, repeated at least once, consisted of three replicates of each *M. xanthus* strain-and-prey combination. The percentage of the wild-type response calculated was used in statistical analyses of the data. Analysis of variance was conducted on raw or transformed data by separation of means with Tukey's HSD using JMP (Version 4, SAS Institute).

## RESULTS

### Predation assays

The susceptibility of *S. marcescens* to predation by *M. xanthus* has been reported elsewhere (Raverdy, 1973; Singh & Yadava, 1976). Recently, we observed that dense lawns of *S. marcescens* formed on otherwise nutrient-free nitrocellulose filters can support the growth of *M. xanthus* laboratory strain DK1622 (data not shown). Presumably, *M. xanthus* was able to lyse cells of *S. marcescens* and use the liberated components for nutritional sustenance. Using this information, we developed the *S. marcescens* lawn predation assay in order to measure *M. xanthus* predation ability under conditions in which the consumption of live prey bacteria is necessary for maintaining growth. In preliminary studies, wild-type and mutant *M. xanthus* cells were spotted at various cell densities ( $1 \times 10^7\text{--}5 \times 10^9$  c.f.u.  $\text{ml}^{-1}$ , corresponding to approximately  $10^6\text{--}10^8$  c.f.u.  $\text{cm}^{-2}$ ) onto *S. marcescens* lawns that we varied in density from approximately  $10^7$  to  $10^9$  c.f.u.  $\text{cm}^{-2}$ ; however, no differences were observed amongst these variables with respect to the final data obtained. We did observe, however, that the time required for reliable and quantifiable differences to arise was shortest – at about 7 days – when  $5 \times 10^9$  c.f.u.  $\text{ml}^{-1}$  ( $10^8$  c.f.u.  $\text{cm}^{-2}$ ) of *M. xanthus* was spotted onto *S. marcescens* lawns of about  $10^8$  c.f.u.  $\text{cm}^{-2}$ ; lower prey densities tended to cause the entire *M. xanthus* colony to initiate development, indicating a lack of sufficient prey-derived nutrients to sustain growth (data not shown). As the *M. xanthus* cells grew out and lysed the *S. marcescens* lawn, the sizes of their swarms were measured to quantify relative predation efficacy. Interestingly, the *M. xanthus* cells in the prey-deprived interior of the swarm were starved, as evidenced by the formation of fruiting bodies in the swarm centre. This indicates that, in this assay, starvation can only be avoided through outward growth into the lawn of prey cells.

**Table 3.** Susceptibility of different prey bacteria to predation by *M. xanthus* strain DK1622 on the streak assay

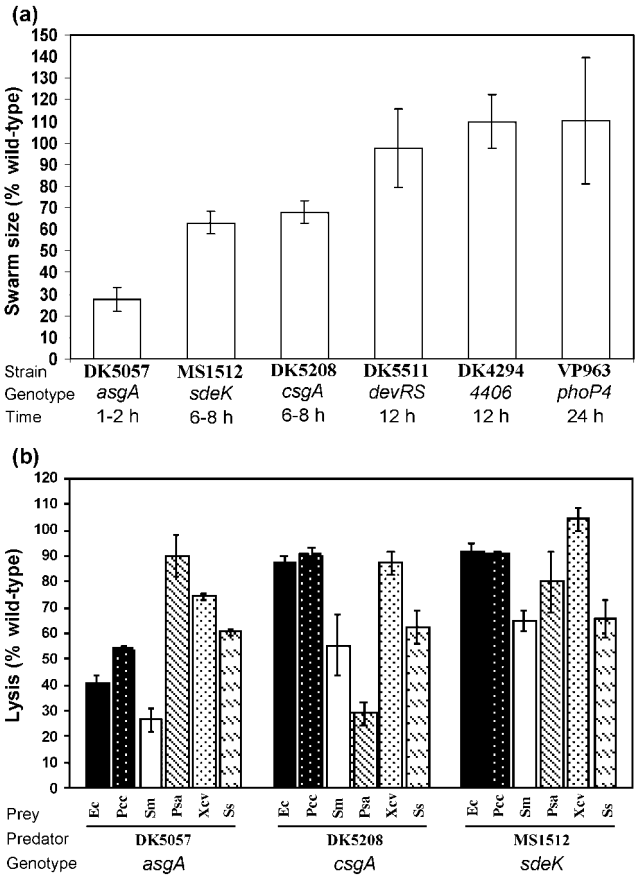
*M. xanthus* cells from threefold replicate cultures were spotted next to a strip of prey cells; the lysis of the strip was measured as described in Methods. Data shown are typical results for replicate experiments that were performed at least twice and found to be reproducible. Refer to Table 2 for a list of prey species and strains.

Prey species	Length (cm) of lysis strip (mean ± SD)
<i>S. marcescens</i> (Sm)	0.74 ± 0.02
<i>Sphing. suberifaciens</i> (Ss)	2.14 ± 0.08
<i>Pseud. syringae</i> (Psa)	2.45 ± 0.38
<i>P. carotovorum</i> (Pcc)	2.63 ± 0.21
<i>X. campestris</i> (Xcv)	2.93 ± 0.12
<i>E. coli</i> (Ec)	3.56 ± 0.14

Additionally, the previously described streak assay (Bull *et al.*, 2002) was used to quantify predation by *M. xanthus* of six different bacterial prey species, including *S. marcescens* (Table 2). Based on multiple experiments conducted using this assay, we observed a gradient of susceptibility to predation among the prey species, with *E. coli* DH5α being the most susceptible, followed by *Xanthomonas campestris* pv. *vitians*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pseudomonas syringae* pv. *alisalensis*, *Sphingomonas suberifaciens*, and finally *S. marcescens* (Table 3).

**Mutations in developmental regulators decrease predation efficiency**

Since predation, like development, depends on an accurate assessment of the local nutrient status, we hypothesized that regulators of early developmental events, that is those at the time when appropriate signalling events are first activated in response to nutrient depletion, would be required for efficient predation. To test this hypothesis, we examined several known developmental mutants for their predation phenotypes. A mutant defective for SdeK, a histidine kinase required for integrating the cellular and population starvation responses (Pollack & Singer, 2001), was examined, as well as mutants in AsgA and CsgA, which are required for A-signal (Kuspa & Kaiser, 1989) and C-signal (Hagen & Shimkets, 1990) production, respectively; these data are presented in Fig. 1(a). These three genes are known to regulate the major early regulatory circuits in *M. xanthus* development. In addition, we assayed mutants for two CsgA- and SdeK-dependent genes, *devRS* (Thöny-Meyer & Kaiser, 1993) and *4406* (Kroos *et al.*, 1986; Pollack & Singer, 2001), which are activated at around 12 h after starvation initiation. Finally, we assayed mutants for a gene named *phoP4* that is required for activating development-specific phosphatase activities 24 h into development (V. D. Pham and others, unpublished results).



**Fig. 1.** (a) Effect of mutations in known developmental genes on the predation ability of *M. xanthus* towards *S. marcescens*, as measured by the lawn assay. Strains are shown (left to right) in the order in which genes are expressed or when phenotypes due to the mutations are manifested during development, with approximate times of occurrence (in hours) into development. Mean values are shown; error bars represent 1SD. (b) Effect of mutations in *asgA* (DK5057), *csgA* (DK5208) and *sdeK* (MS1512) on *M. xanthus* predation of various bacterial prey species, as measured by the streak assay. Mean values are shown; error bars represent 1SD. Within each of the three experiments (same predator strain, different prey strains), bars with overlapping standard deviation lines were determined to be not significantly different ( $P > 0.05$ ) according to Tukey's HSD test. Abbreviations: Ec, *Escherichia coli*; Pcc, *Pectobacterium carotovorum* subsp. *carotovorum*; Sm, *Serratia marcescens*; Psa, *Pseudomonas syringae* pv. *alisalensis*; Xcv, *Xanthomonas campestris* pv. *vitians*; Ss, *Sphingomonas suberifaciens*.

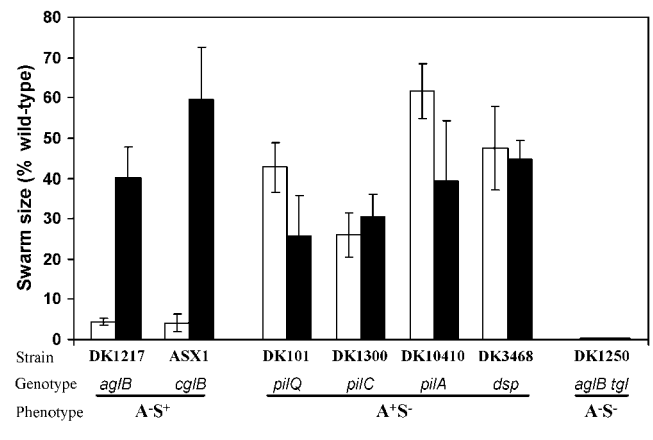
The lawn assay results for these developmental regulatory mutants are shown in Fig. 1(a). We observed that the *asgA* mutant strain DK5057 had the strongest defect in predation of *S. marcescens*, at 27 % of wild-type (Fig. 1a). In contrast to the A signal, which is generated at around 1–2 h post-starvation initiation, the later C signal, which governs spatial positioning of cells at aggregation foci and is produced at

around 6–8 h post-starvation, had a milder predation defect: strain DK5208 (*csgA*:: $\Omega$ 205) had a predation ability of 67 % compared to wild-type. This was very similar to the 64 % defect observed for strain MS1512 ( $\Delta$ *sdeK1*), which contains a deletion of *sdeK*. Neither insertion mutations in *devR* (DK5511) and *4406* (DK4294), nor a deletion of *phoP4* (VP963), caused measurable defects (Fig. 1a).

The lawn assay data for the global regulators are similar to the streak assay results when *S. marcescens* is used as the prey species (Fig. 1b). For the streak assay, predation by the *asgA* strain is also 27 % of wild-type, and both the *csgA* and *sdeK* mutants had about 60 % wild-type predation efficiencies (Fig. 1b). When the same mutants were assessed for predation ability of five other prey species, the results revealed prey-specific effects (Fig. 1b). Whereas the *asgA* mutant suffered defects of between 27 and 74 % on five of the prey species analysed (*Pseud. syringae* had a minimal defect of about 90 % compared to wild-type), the *csgA* and *sdeK* mutants showed significant defects only for predation on *Pseud. syringae*, *S. marcescens*, and *Sphing. suberifaciens*. However, while *csgA* and *sdeK* mutants showed quantitatively similar defects when tested on *S. marcescens* and *Sphing. suberifaciens* (about 60–70 % of wild-type), predation of *Pseud. syringae* was affected the most by the *csgA* mutation (about 30 % of wild-type), while the defect due to the *sdeK* mutation was relatively mild (about 80 % of wild-type).

### Several Asg genes are required for wild-type predation

Based on the observed defect of the *asgA* mutant in our predation assays, we were interested in determining to what extent A signalling may be involved in predation. To this end, the predation phenotypes of mutants in three



**Fig. 2.** Effect of mutations in *M. xanthus* adventurous (A) and social (S) motility genes on predation ability, as measured by the lawn predation assay. White bars represent predation assay results, while black bars represent measurements of the mobility of mutants on nitrocellulose in the absence of prey. Mean values are shown; error bars represent 1 SD.

additional Asg genes – *asgB480* (Kuspa & Kaiser, 1989), *asgC767* (Kuspa & Kaiser, 1989) and *asgE*:: $\Omega$ 5003 (Garza *et al.*, 2000) – were determined. Like the *asgA* mutant, mutations in *asgC* and *asgE* caused a defect in predation (Table 4). However, the *asgB* mutant showed no predation defect, which suggests that the A signal is not necessary for predation and that some other function of the AsgACE regulon plays an important role in regulating the predatory process.

### Adventurous motility mutants are defective for predation

*M. xanthus* is a mobile predator, with two distinct motility systems that are termed adventurous (A) and social (S). To assess whether either motility system is essential for predation, different mutants with A<sup>−</sup> and/or S<sup>−</sup> motility phenotypes were assayed (Fig. 2). Because mutations that impede mobility might affect swarm expansion on nitrocellulose (and hence affect the lawn assay results), we compared the relative swarm sizes of each mutant spotted onto nitrocellulose in the presence of freely accessible nutrients, in other words CTTYE (Fig. 2, black bars), to those spotted in the presence of prey bacteria (Fig. 2, white bars). The predation defect due to factors other than motility can therefore be gauged by the difference in relative swarm sizes on prey-plus and prey-minus (i.e. CTTYE-soaked) membranes.

When this was done, the results showed a striking predation effect for both of the A<sup>−</sup> mutants examined. While the defect ascribable to motility alone (that is, in the absence of prey) is between 40 % and 60 % compared to wild-type, the relative A<sup>−</sup> swarm sizes decreased to 5 % of wild-type when prey was present (Fig. 2). Therefore, the data indicate that the A<sup>−</sup>

**Table 4.** Predatory phenotype of Asg and Frz Mutants

*M. xanthus* mutants were assessed by the lawn assay (as described in Methods) for predation efficiency relative to their respective parental wild type strain. Data shown are typical results for four-fold replicate experiments that were performed at least twice and found to be reproducible. Refer to Table 1 for a list of strains.

Relevant mutation (strain)	Swarm size as a percentage of wild-type (mean $\pm$ SD)
<i>asgA476</i> (DK5057)	27.6 $\pm$ 5.64
<i>asgB480</i> (DK480)	107.8 $\pm$ 15.63
<i>asgC767</i> (DK767)	36.8 $\pm$ 9.79
<i>asgE</i> :: $\Omega$ 5003 (MS2021)	20.8 $\pm$ 1.32
$\Delta$ <i>frzA</i> (DZ4478)	15.5 $\pm$ 2.34
$\Delta$ <i>frzB</i> (DZ4479)	38.2 $\pm$ 5.79
$\Delta$ <i>frzE</i> (DZ4481)	18.2 $\pm$ 4.11
$\Delta$ <i>frzF</i> (DZ4483)	26.7 $\pm$ 1.73
$\Delta$ <i>frzZ</i> (DZ4484)	30.2 $\pm$ 7.54

defect for predation and motility together was significantly greater than the A<sup>-</sup> defect due to motility alone. This demonstrates that the A-motility system is important for predation.

In contrast, the decreased swarm sizes observed for S-motility mutants can be wholly attributed to their decreased motility on the substrate (Fig. 2). This was true for mutations causing abnormal assembly of both type IV pili and fibrils, two different extracellular components that are essential for S motility (for a review, consult Spormann, 1999). These results showed that, by our assay, S<sup>-</sup> mutations by themselves do not cause a predation phenotype. However, because S motility is more effective on soft agar (Shi & Zusman, 1993), our assay cannot entirely rule out that S motility plays a predation-dependent role on soft and wet surfaces. What the results do show, though, is that when both the A- and S-motility systems are knocked out, as in strain DK1250, *M. xanthus* can neither move nor prey on *S. marcescens*. This defect was even more severe than those for the A<sup>-</sup> mutants. Thus, even if the S-motility system may not be essential for predation by itself, *M. xanthus* swarms cannot expand and utilize *S. marcescens* as a food source without at least one functioning motility system.

### Frz mutants are defective in predation

To complete our studies on the role of motility in predation, we determined the predation phenotype of several Frz mutants by the lawn assay. In *M. xanthus*, the Frz proteins control the reversal frequency for cell movement and are homologous to the Che proteins of *E. coli* (Blackhart & Zusman, 1985; McBride *et al.*, 1989; McCleary & Zusman, 1990). As a result, it has been proposed that the Frz system is involved in modulating directed cell movements in *M. xanthus* (Kearns & Shimkets, 1998; Shi *et al.*, 1993), and thus could play an important role in predation. Mutants in five *frz* genes were assayed, along with the respective wild-type parent (Table 4). In all cases, predation was decreased by between 62 and 85 % of wild-type, which strongly suggests a role for tactic behaviour in predation. Previous studies demonstrated that *frz* mutants, like S-motility mutants, have a defect in swarming on soft and wet surfaces, such as 0.3 % agar, and only a mild defect on firm surfaces, such as 1.5 % agar (Bustamante *et al.*, 2004). When tested on nitrocellulose in the absence of prey, swarm sizes for *frz* mutants were found to differ only to a negligible extent (10 % or less) from the wild-type control (data not shown). Therefore, the observed predation defects of the *frz* strains on the firm surface of the lawn assay are due to a defect in tactic functions rather than in motility.

## DISCUSSION

Numerous types of evidence exist for predatory relationships among micro-organisms, suggesting that microbial competition for limiting resources is marked by food-web relationships analogous to those seen in the macroscopic world (for reviews, consult Alexander, 1981; Casida, 1988;

Hahn & Höfle, 2001; Martin, 2002). Since the lysis of *Micrococcus* sp. by the myxobacterium *Chondromyces crocatus* was first reported (Pinoy, 1921), myxobacteria have been recognized as important micropredators that possess the capacity for lysing diverse prey species (for a review, consult Rosenberg & Varon, 1984).

Conceptually, predation can be thought of as a behaviour that, like development, is dependent on a series of inter-linked processes required for myxobacterial nutrient scavenging. Among these are systems for hunger alert (signalling), predator-to-predator communication (quorum sensing), prey detection (chemotaxis or chemokinesis), prey tracking (motility), production of killing implements (biolytic compounds) and feeding (lysis of prey and nutrient absorption). While some of these processes have been studied individually, the overall contribution of relevant genetic components to predation has received scant attention in the past.

Here, we demonstrated the involvement of some of these processes in predation by assessing the predation phenotypes of relevant mutants. We developed a simple assay to quantify predation by *M. xanthus*, which is easily the most well-characterized myxobacterium. The assay measures predation under conditions in which the consumption of live prey is essential for maintaining growth. This is in contrast to the previously reported streak assay, which measures predation of prey in the presence of limited biotic nutrients. Under the conditions of the streak assay, variations in sensitivity to predation by *M. xanthus* were detected among different prey bacteria (Table 3). The fact that *S. marcescens* was the species least sensitive to lysis in the streak assay suggested that using *S. marcescens* to score predation ability in the lawn assay would give the fewest false positive results. However, it is equally possible that the gradient of prey susceptibility observed is specific for the conditions of the streak assay, perhaps because the presence of biotic nutrients limits predation of certain prey bacteria. Regardless of this, the crucial point is that two distinct assays, measuring predation under different conditions, are available to monitor and quantify changes in the predation ability of a variety of *M. xanthus* strains. Since predation is complex and results may vary depending on the method used, both assays were employed in the study reported here. Strikingly, for those mutants for which data were gathered by both methods, the results were very similar. This implies that relevant pathways important for predation may be turned on in *M. xanthus* whenever prey is available, regardless of whether alternative sources of sustenance (e.g. in the form of limited biotic nutrients) are present.

### Predation and development

The most severe predation defect was observed for strains with mutations in three known A-signal genes, *asgA*, *asgC* and *asgE* (Fig. 1, Table 4). *AsgA* is a signal transduction protein required for generating the quorum-sensing A signal, which informs starving *M. xanthus* cells of whether

the colony has reached a threshold cell density to initiate development (Kuspa *et al.*, 1992). Mutations in *asgA* cause decreased A-signal production and aborted development (Kuspa & Kaiser, 1989). A defect in AsgC, a homologue of the vegetative sigma factor RpoD that is predicted to have a variety of effects on gene expression (Davis *et al.*, 1995; Hernandez & Cashel, 1995), is similarly defective for A-signal production and development (Kuspa & Kaiser, 1989). AseE, which is homologous to amidohydrolases that cleave heterocyclic compounds (Garza *et al.*, 2000), is believed to function downstream of AseA: while extracts from *aseE* cells can partly rescue the *asgA* sporulation defect, the reciprocal rescue of *aseE* cells by *asgA* cells does not occur.

Unlike the *asgA*, *asgC* and *aseE* strains examined, a strain defective for AseB, which is required for generating at least two extracellular proteases that have been implicated in generating the A signal (Plamann *et al.*, 1992), behaved like wild-type in our assay. This implies that the A signal itself is not required for predation, but that other genes under the control of AseA and AseC, such as *aseE*, are involved in predatory behaviours. These results support our previous hypothesis that A signalling is complex and may represent a series of signals and proteins under the control of AseA (Garza *et al.*, 2000).

Somewhat less pronounced, though consistently detected, predation phenotypes were also detected for *csgA* and *sdeK* mutants (Fig. 1a, b). The defects, at around 60–70 % of wild-type levels, were almost identical for these two regulators (within experimental error). CsgA and SdeK are active at the same time, and function in parallel pathways that converge on common targets (Pollack & Singer, 2001). However, mutations in *devR* and *4406*, components that act downstream of both pathways (Kroos *et al.*, 1986), did not exhibit measurable defects (Fig. 1a). One interpretation is that the predation function of the CsgA and SdeK pathways is mediated through other components not yet identified. We predict that such hypothetical components are likely turned on soon after SdeK and CsgA become active, since it appears that the earlier a regulator functions in development, the more severe the predation defect. This suggests a model whereby early developmental components regulate processes that are also important for predation of *S. marcescens*, such as detecting nutrient availability, while later components function in pathways that are specific for development. Consistent with this model is the observation that PhoP4 is also not important for predation (Fig. 1a), although it is an essential regulator of developmental phosphatases that are activated only after 24 h into development (V. D. Pham and others, unpublished results).

The above model is based on data collected for predation of *S. marcescens*. When *M. xanthus* predation of other prey bacteria was tested using the streak assay, prey-specific responses among the genetic mutants became evident (Fig. 1b, Table 3) which suggests a high degree of complexity for the genetic circuitries that regulate predation behaviour. Perhaps predation of different prey species is

effected through different pathways, each of which could regulate specialized predation mechanisms mediated by differential production of antimicrobials or by hydrolytic enzymes that would affect potential prey species differently. Possessing multiple predation pathways would allow *M. xanthus* to make specific, tailored responses depending on the availability of different subsets of prey species in the soil.

## Predation and motility

Most methods for isolating bacteriolytic myxobacteria depend on the movement of myxobacterial cells from the originating inoculation material – usually a patch of soil or a dung pellet – onto an agar substrate containing prey bacteria (for a review, consult Dawid, 2000). This observation suggests an important role for motility in predation. Here, we directly examined the role of the A- and S-motility systems, as well as the Frz chemotaxis-like system, by assaying relevant mutants for predatory ability.

First, we tested the non-motile strain DK1250 (*aglB1 tgl-1*), which has a mutation in both the A- and S-motility systems (Hodgkin & Kaiser, 1979a). Consistent with the prediction that motility is an important predation factor, these A<sup>−</sup> S<sup>−</sup> cells were unable to prey on feeder bacteria (Fig. 2). Among the S-motility mutants examined, all mutants displayed significantly reduced swarm sizes; however, these defects were very similar to those attributed solely to the reduced mobility of these mutants on nitrocellulose in the absence of prey (Fig. 2). Thus, the conclusion is that, under the conditions of our assay, S motility is not important for predation.

In contrast, the A-motility system does appear to be essential for predation. The two A<sup>−</sup> mutants examined had defects in predation of between 35 and 55 % after motility-associated effects were factored out. The most well-characterized A-motility gene is *cglB*, which appears to code for an outer-membrane lipoprotein important for regulating reversal frequency (Rodriguez & Spormann, 1999; Spormann & Kaiser, 1999). Interestingly, the *cglB* mutant is able to secrete the polyelectrolyte gel thought to power A motility, though its uniform deposition around the cell – rather than exclusively from the poles – suggests an anatomical defect to which its A<sup>−</sup> phenotype can be attributed (Wolgemuth *et al.*, 2002).

The A-motility system powers movement of individual cells along slime trails, and it is tempting to hypothesize that the observed defect in predation could be ascribed to an inability to track chemotactic gradients laid down by prey bacteria. Our observation that the Frz system is required for predation supports this hypothesis. Different investigators working on various myxobacterial species, including *M. xanthus*, recorded biased movement towards food bacteria (Daft & Stewart, 1973; Dworkin, 1983; Kühlwein & Reichenbach, 1968). However, chemotaxis during vegetative growth is not well-characterized in *M. xanthus*,

and models have been advanced suggesting that *M. xanthus* gliding motility is too slow to allow for chemotaxis of small, fast-diffusing molecules, though chemokinesis of such molecules may be accommodated (Dworkin & Eide, 1983; Shi & Zusman, 1994). Recently, data were presented for the directed movement of *M. xanthus* cells in response to fatty acid chemoattractants (Kearns *et al.*, 2001), which suggests that these cells are able to track large molecules with slow diffusion rates. Since the detection of prey or prey trace signatures may be an integral part of predatory behaviour, much could be learned about chemotaxis in *M. xanthus* by assessing the predation abilities of relevant genetic mutants in greater detail. It has been reported that there are eight clusters of genes encoding Che-like components in *M. xanthus* (The Institute for Genomic Research). Here, we reported the importance of one of these systems, the Frz system, to predation, but future studies will be needed to determine if additional chemotaxis-like systems also have a role to play.

### Concluding remarks

The data presented here demonstrate that predation in *M. xanthus* is dependent upon genes required for predation-associated processes, such as signalling and motility. However, the components involved may not play uniformly significant roles for predation of all susceptible prey species, and many more additional predation factors almost certainly remain to be identified. It is likely that these will include strain-specific biolytic factors useful for predation of different subsets of prey, as well as conserved global regulators instructing predation pathways common to all strains. The discovery of all these components is a prerequisite for a more thorough understanding of the genetics of *M. xanthus* predation.

Although bacterial predation by myxobacteria has been demonstrated in soil microcosms (Casida, 1980; Liu & Casida, 1983), little fundamental information about factors influencing predation in nature is available. In this manuscript, we have described the interaction between myxobacterial predators – in the form of developmental mutants – and bacterial prey strains, thus providing the first information about developmental factors that influence predation. Although we cannot directly extrapolate these results to natural systems, they do provide us with interesting hypotheses to test in soil systems. Therefore, we have begun to evaluate the role of the mutants with the most pronounced phenotypes in assays evaluating inhibition and predation of plant pathogens in soil systems. For example, our preliminary data indicate that developmental mutants can reduce the control of diseases caused by fungal pathogens (C. T. Bull, unpublished data). In order to clearly describe predation and antagonism in terrestrial ecosystems, we are developing additional tools, including culture-independent enumeration techniques (Pepper *et al.*, 2004), to study myxobacterial predation and antibiosis in soil systems.

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